Pyruvate and Ketone-Body Transport across the Mitochondrial Membrane

EXCHANGE PROPERTIES, pH-DEPENDENCE AND MECHANISM OF THE CARRIER

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The effects of exchangeable ions and pH on the efflux of pyruvate from preloaded mitochondria are reported. Efflux obeys first-order kinetics, and the stimulation of efflux by exchangeable ions such as acetoacetate and lactate obeys Michaelis–Menten kinetics. The apparent K_m value $\pm$ s.e. for acetoacetate was 0.56 ± 0.14 mM ($n = 5$) and that for lactate 12.3 ± 2.3 mM ($n = 6$). The V_{max} values $\pm$ s.e. at 0°C were 16.2 ± 2.0 and 21.9 ± 2.7 nmol/min per mg of protein. The exchange of a variety of other substituted monocarboxylates was also studied. Efflux was also stimulated by increasing the external pH. The data gave a pK for the transport process of 8.35 and a V_{max} of 3.31 ± 0.14 nmol/min per mg. The similarity of the V_{max} values for various exchangeable ions but the difference of this from the V_{max} in the absence of exchangeable ions may indicate that transport of pyruvate occurs with H^+ and not in exchange for an OH^- ion. The inhibition of transport by α -cyano-4-hydroxycinnamate took several seconds to reach completion at 0°C . It is proposed that inhibition occurs by binding to the substrate site and subsequent reaction with an -SH group on the inside of the membrane. The inhibitor can be displaced by substrates that can also enter the mitochondria independently of the carrier and so compete with the inhibitor for the substrate-binding site on the inside of the membrane. A mechanism for transport is proposed that invokes a transition state of pyruvate involving addition of an -SH group to the 2-carbon of pyruvate. Evidence is presented that suggests that ketone bodies may cross the mitochondrial membrane either on the carrier or by free diffusion. The physiological involvement of the carrier in ketone-body metabolism is discussed. The role of ketone bodies and pH in the physiological regulation of pyruvate transport is considered.

Since the proposal that pyruvate enters the mitochondrion on a specific carrier (Papa *et al.*, 1971) and the subsequent support for this by the use of the specific inhibitor α -cyano-4-hydroxycinnamate (Halestrap & Denton, 1974; Halestrap, 1975), a considerable amount of work has been published on the properties of this transport system (Papa & Paradies, 1974; Halestrap, 1975, 1976; Halestrap & Denton, 1975; Paradies & Papa, 1975, 1976; Titheradge & Coore, 1975, 1976; Mowbray, 1975). Within this collection of data there is considerable divergence of results, which reflects the various techniques used to study pyruvate transport. In this paper experiments are presented that seek to clarify and extend our knowledge of the pyruvate carrier. The exchange properties of the carrier are contrasted with the net flux of pyruvate through the carrier, and the mode of inhibition by α -cyano-4-hydroxycinnamate is studied in more depth. From these studies a mechan-

ism for the carrier is proposed that suggests that net flux of pyruvate occurs with a H^+ rather than in exchange for an OH^- ion. The relevance of this to the physiological situation and the potential control of pyruvate transport is considered. The ability of the carrier to transport ketone bodies is also studied in relation to the situation occurring physiologically, and it is concluded that at the normal cellular concentrations of these metabolites, acetoacetate may use the pyruvate carrier, as well as diffusion, to cross the mitochondrial membrane, whereas β -hydroxybutyrate probably diffuses as the free acid. In the ketogenic situation the exchange of ketone bodies leaving the mitochondria with pyruvate entering for gluconeogenesis may be important.

Experimental

Materials

Except where stated below, all chemicals and biochemicals were obtained from the sources given

Abbreviations used: Mops, 4-morpholinepropane-sulphonic acid; Meps, 4-morpholine-ethanesulphonic acid.

previously (Halestrap, 1975). All radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. Compound UK 5099 [α -cyano- β -(1-phenylindol-3-yl)acrylate] was a gift from Pfizer, Sandwich, Kent, UK Acetoacetate (lithium salt) was obtained from Sigma (London) Chemical Co., London S.W.6, U.K., and D- β -hydroxybutyrate dehydrogenase (EC 1.1.1.30) was from Boehringer Corp. (London) Ltd., London W.5, U.K. Mitochondria were prepared as described by Halestrap (1975).

Methods

Pyruvate-efflux experiments. Mitochondria (prepared at pH 7.5) were incubated for 4 min at approx. 4 mg/ml in 7 ml of medium (125 mM-KCl/20 mM-Mops/10 μ M-rotenone, pH 6.7) containing 0.5 mM-[2- 14 C]pyruvate (0.05 μ Ci/ml) and [6,6'- 3 H]sucrose (0.5 μ Ci/ml) at 7°C. Samples (5 $\times$ 1 ml) were centrifuged at 10000g for 1 min at 7°C in an Eppendorf 3200 centrifuge and the supernatants removed and acidified with HClO₄ to 2%. The pellets were immediately stored on ice to avoid pyruvate metabolism. At zero time, incubation buffer at 0°C containing the relevant exchangeable ion was added to the pellet and the tube rapidly vortex-mixed. Efflux was terminated at the required time by addition of 2.5 mM- α -cyano-4-hydroxycinnamate and the mitochondria were sedimented by centrifugation as above. The supernatant was discarded and the pellet analysed for intramitochondrial 14 C as described previously (Halestrap, 1975). Control experiments were performed by adding 2.5 mM- α -cyano-4-hydroxycinnamate with the exchangeable ion was added after the medium and inhibitor had been added. These experiments allowed an accurate estimate of the 'dead time' (the time taken for the inhibitor to inhibit efflux totally). This dead time is about 5 s at 0°C and at pH 6.7, but appears to become longer at higher pH values. This can be explained by the data below, which suggest that the inhibitor must cross the mitochondrial membrane to inhibit. Since α -cyanocinnamate derivatives cross the mitochondrial membrane as free acids (Halestrap, 1975), the rate of this process will be lower at higher pH values.

Direct measurement of the rate of pyruvate uptake by mitochondria was measured under conditions of an artificially induced pH gradient as described by Halestrap (1975). Details are given in the legends to the relevant Figures. O₂ uptake by respiring mitochondria and the carboxylation of pyruvate by mitochondria were measured as described previously (Halestrap, 1975; Halestrap & Denton, 1975) and in the legends to the Figures and Tables.

Pyruvate and lactate were assayed as described by Halestrap & Denton (1974). Acetoacetate and D- β -hydroxybutyrate were assayed with D- β -hydroxy-

butyrate dehydrogenase (Mellanby & Williamson, 1963; Williamson & Mellanby, 1963).

Results and Discussion

Exchange kinetics of the pyruvate transporter

The time course of the efflux of pyruvate at 0°C from preloaded mitochondria is shown in Fig. 1(a) and 1(b). At pH 6.7, if no exchangeable ion is present, the rate of loss of pyruvate is barely measurable. However, if an exchangeable ion such as pyruvate or acetoacetate is added or the pH of the medium is raised, a rapid and extensive loss of pyruvate is observed which follows first-order kinetics (Fig. 2). That the loss of 14 C measured in these experiments represents enzymically assayable pyruvate has been confirmed before (Halestrap & Denton, 1974; Halestrap, 1975). Extrapolation of the data of Fig. 2 shows that there is a dead time of about 5 s at this temperature for the action of α -cyano-4-hydroxycinnamate used to stop the reaction. This does not permit accurate kinetic analysis of the efflux of very rapidly exchangeable ions such as pyruvate and chloroacetate, but lower efflux rates were able to be analysed by first-order rate kinetics. From such data it was established that the exchange of acetoacetate and L-lactate observed Michaelis-Menten kinetics (Fig. 3), with apparent K_m values $\pm$ s.e. of 0.56 ± 0.14 mM ($n = 5$) and 12.3 ± 2.3 mM ($n = 6$) and V_{max} values $\pm$ s.e. of 16.2 ± 1.9 and 21.9 ± 2.7 nmol/min per mg of mitochondrial protein respectively. Thus, in agreement with Paradies & Papa (1975), it is concluded that exchangeable ions of greatly differing affinities for the pyruvate carrier exhibit the same maximum rate of exchange. The rate of exchange of a variety of other exchangeable ions (Halestrap, 1975) and approximate K_m values are given in Table 1. These values are consistent with those of Paradies & Papa (1975, 1976), who used rapid centrifugation techniques rather than the inhibitor-stop method used here. In contrast with reports of others (Mowbray, 1975; Titheradge & Coore, 1975), neither malate nor palmitoylcarnitine are readily exchangeable under these conditions, which agrees with the results of Paradies & Papa (1976). However, malate does show some exchange, but the effect is probably too small to be considered of great physiological importance, as suggested by Titheradge & Coore (1976). Of more interest physiologically are the effects of ketone bodies and pH and these are considered in more detail below.

Effect of pH on pyruvate transport

The effects of altering the pH of the exchange medium are shown in Fig. 4. The data follow a pK curve that gives a pK value of 8.35 (95% confidence limits 8.20 to 8.45) and a maximum rate of transport of 3.31 ± 0.14 nmol/min per mg ($n = 10$). It is note-

Fig. 1. Time course of efflux of pyruvate from preloaded mitochondria

Rat liver mitochondria (approx. 4 mg/ml) were loaded with pyruvate as described under 'Methods' and efflux was initiated by addition of medium (125 mM-KCl/10 mM-Mops/10 mM-Tris/HCl/10 mM-Meps/10 μ M-rotenone) at pH 6.7 (a) or the pH indicated (b). Other additions were made in (a) as indicated. ●, None; ▲, 75 μ M-palmitoylcarnitine; ■, 0.5 mM-L-malate; △, 0.5 mM-L-lactate; □, 0.5 mM-acetoacetate; ▼, 0.5 mM-pyruvate; ○, 0.5 mM-pyruvate added after preincubation for 1 min with 2.5 mM- α -cyano-4-hydroxycinnamate. Efflux was terminated by addition of 2.5 mM- α -cyano-4-hydroxycinnamate and the pyruvate remaining in the mitochondria analysed as described under 'Methods'. Values of the pyruvate remaining are expressed as percentages of the initial pyruvate (2.3 nmol/mg) inside the mitochondria.

Fig. 2. First-order plot of the efflux of pyruvate from preloaded mitochondria

The experimental procedure was the same as that for Fig. 1. The final extent of efflux (P_f), the rate constant (k) and the 'dead time' (x) were estimated by least-squares regression to the equation:

$$(P_i - P_f) = (P_0 - P_f)e^{-k(t+x)}$$

where P_i , P_0 and P_f are the amounts of pyruvate remaining in the mitochondria at final equilibrium

worthy that the rate of efflux (about 0.65 nmol/min per mg at 0°C) at pH 7.6 is similar to the rate of pyruvate uptake (1.9 nmol/min per mg) at 6°C measured (as described by Halestrap, 1975) at an internal pH of 7.6, when the activation energy of the transporter (113 kJ/mol; Halestrap, 1975) is considered.

Two consequences of the effect of pH on the pyruvate transporter are apparent. First, the net rate of pyruvate transport into the mitochondria will be very sensitive to changes in the matrix pH within the physiological range. Secondly, the fact that the maximum rate of pyruvate efflux in the absence of an exchangeable ion is considerably lower than that obtainable in the presence of an exchangeable ion suggests that pyruvate does not normally exchange with an OH^- ion. Rather it would appear that the pyruvate enters with a H^+ as illustrated in Scheme 1. From the V_{max} values derived for exchange and net

(i.e. when no further efflux occurs), initially and time t respectively. The initial rates [$k(P_0 - P_f)$] were calculated at 6.1 nmol/min per mg for 0.5 mM-acetoacetate (■), 1.4 nmol/min per mg for 0.5 mM-lactate (▲) and 0.2 nmol/min per mg for 75 μ M-palmitoylcarnitine (●).

flux of pyruvate above, it appears that the maximum rate of process (a) (i.e. when all the carrier is as [carrier-B]) is 3.31 nmol/min per mg at 0°C, and that

Fig. 3. Concentration-dependence of acetoacetate and L-lactate exchange with mitochondrial pyruvate

Rates of pyruvate efflux at 0°C were determined as described in Figs. 1 and 2 in the presence of various concentrations of acetoacetate (■) and L-lactate (●). The $K_m \pm \text{s.e.}$ for acetoacetate was calculated to be $0.56 \pm 0.14 \text{ mm}$ and that for lactate $12.3 \pm 2.3 \text{ mm}$. The V_{max} values $\pm \text{s.e.}$ were 16.2 ± 2.0 and $21.9 \pm 2.7 \text{ nmol/min per mg}$ respectively. Values were calculated by least-squares regression of the Michaelis-Menten equation.

Fig. 4. Effect of pH on the efflux of pyruvate from preloaded mitochondria

Efflux was studied as described in Fig. 1 and the rates of efflux were calculated by first-order plots as in Fig. 2. Least-squares regression of the curve gave a $V_{\text{max}} \pm \text{s.e.}$ for transport of $3.3 \pm 0.14 \text{ nmol/min per mg}$ and an apparent $K_m \pm \text{s.e.}$ for OH^- of $2.2 \pm 0.25 \mu\text{M}$, i.e. a pK of 8.35 (95% confidence limits 8.20–8.45).

Table 1. Rates of exchange of a variety of substrates on the pyruvate carrier

Rates of pyruvate efflux were determined as described in Figs. 1 and 2. The approximate apparent K_m values were calculated assuming that all substrates exhibit the same V_{max} of $19 \text{ nmol/min per mg}$. This appears to be true for all small carboxylates tested (Fig. 3, and Paradies & Papa, 1975). It may not be true for carboxylates with large hydrophobic side chains capable of hydrophobic interactions which might slow down the rate of release of the substrate; this is suggested by the difference in the apparent K_m for phenylpyruvate of 9.7 mm from its K_1 of 1.8 mm (Halestrap, 1975). Data for acetoacetate and L-lactate are taken from Fig. 3. The remaining data were obtained from time courses containing six time points per substrate.

Substrate	Concentration (mm)	Rate of pyruvate efflux (nmol/min per mg)	Approximate apparent K_m (mm)
Pyruvate	0.5	10	<0.4
Acetoacetate	∞	16.2 ± 2.0	0.56 ± 0.14
L-Lactate	∞	21.9 ± 2.7	12.3 ± 2.3
DL- β -Hydroxybutyrate	0.5	1.6	5.6
Bromopyruvate	1.0	9.9	1.0
Hydroxypyruvate	1.0	7.4	1.61
Oxamate	1.0	4.2	3.6
Phenylpyruvate	1.0	1.8	9.7
2-Oxo-4-methylpentanoate	1.0	1.7	10.4
2-Oxo-3-methylbutyrate	0.5	1.9	4.6
Monochloroacetate	0.5	10	<0.4
Dichloroacetate	0.5	6.0	1.1
Glyoxylate	1.0	0.9	19.8
L-Malate	0.5	0.5	18.4

Scheme 1. Proposed model for the simultaneous uptake of a proton with a pyruvate anion on the pyruvate carrier

Fig. 5. Time course of pyruvate uptake into mitochondria and the rate of inhibition by α -cyano-4-hydroxycinnamate

Mitochondria prepared at pH 7.5 were added at time 0 to medium (125 mM-KCl/20 mM-Mops/10 μ M-rotenone/0.5 μ Ci of [6,6- 3 H]sucrose/ml, pH 6.7) at 7°C to give a final concentration of about 4 mg of protein/ml. The medium also contained [2- 14 C] pyruvate (0.25 μ Ci/ μ mol) at 0.05 mM (●, ○), 0.2 mM (▲, △) and 0.5 mM (■, □), and (open symbols) 4 μ M- α -cyano-4-hydroxycinnamate. The mitochondria were stirred in a water-jacketed container at 7°C and 1 ml samples removed and rapidly mixed with 2.5 mM- α -cyano-4-hydroxycinnamate at the times indicated. After centrifugation (10000g), the mitochondrial pellet was analysed for 14 C and 3 H as described by Halestrap (1975) and the uptake of pyruvate calculated.

Attempts to block an amino group with pyruvaldehyde have not been successful.

Mechanism of inhibition by α -cyano-4-hydroxycinnamate

Halestrap (1975, 1976) has suggested that α -cyano-4-hydroxycinnamate inhibits pyruvate transport non-competitively by attacking an essential -SH group. However, Paradies & Papa (1975) report that the inhibition is competitive. The cause of this discrepancy may be explained by the different techniques used. Fig. 5 shows that if mitochondria are added to the medium containing pyruvate in the presence of α -cyano-4-hydroxycinnamate, the time course of uptake is not a simple first-order one. Rather initially uptake is not inhibited, but inhibition develops after about 10 s. The inhibitor takes time to act, as in the efflux experiments, where a dead time for inhibition was observed. The technique used by Paradies & Papa (1975) is one of rapid centrifugation of mitochondria through a layer of medium containing pyruvate and inhibitor. The estimated exposure of the mitochondria to the substrate and inhibitor is only about 15 s (Papa & Paradies, 1974). Thus Paradies and Papa are studying the kinetics of inhibition under conditions where full inhibition is not achieved, whereas under the conditions I used, preincubation with inhibitor was performed (Halestrap, 1975). It would seem highly probable that α -cyano-4-hydroxycinnamate inhibits pyruvate transport in two phases, firstly by binding competitively to the pyruvate binding site and then by attacking the essential thiol group. Paradies & Papa (1975) are essentially studying the first phase of inhibition. However, the attack of thiol groups by α -cyano-4-hydroxycinnamate is freely reversible (Halestrap, 1976) and so if the above-mentioned mechanism is correct, one would still expect the inhibitor to be displaced by excess pyruvate, unless the pyruvate were unable to reach the inhibited carrier. Where mitochondria are preincubated with inhibitor, if the

for process (b) (i.e. when all the carrier is as [carrier-BH $^+$ pyruvate $^-$]) is about 19 nmol/min per mg.

The pK of the group B is about 8.35 and could therefore be a histidine or side-chain amino group.

inhibitor attacks the -SH group on the inside of the membrane and traps the carrier there, then pyruvate will be unable to reach the inhibited carrier and the inhibition will appear non-competitive. Two experimental techniques have been used to demonstrate this. First, non-metabolizable exchangeable ions that can enter the mitochondria independently of the carrier should relieve inhibition by α -cyano-4-hydroxycinnamate and its analogues. In Fig. 6 the effect of monochloroacetate and lactate on the K_i of α -cyano-4-hydroxycinnamate for pyruvate transport in liver mitochondria oxidizing pyruvate is shown. A Dixon plot of the rate of pyruvate uptake against inhibitor concentration allows the calculation of the K_i for inhibitor and the V_{max} of pyruvate transport under metabolizing conditions. In the

Fig. 6. Effects of monochloroacetate and L-lactate on the K_i for α -cyano-4-hydroxycinnamate of rat liver mitochondria metabolizing pyruvate

Rat liver mitochondria (approx. 3 mg/ml) were dispersed in medium (125 mM-KCl/10 mM-Mops/10 mM-Tris/HCl/10 mM-Meps/4 mM-sodium pyruvate/0.5 mM-potassium L-malate/2.5 mM-potassium phosphate/5 mM-MgCl₂/2 μ M-carbonylcyanide *p*-trifluoromethoxyphenylhydrazine, pH 7.0) at 0°C. Metabolism was started by addition of 1 ml samples to shaking incubation flasks at 37°C containing α -cyano-4-hydroxycinnamate at the concentration indicated and other additions as shown and terminated after 15 min by addition of 0.1 ml of 20% (w/v) HClO₄. Uptake of pyruvate, which was linear for at least 20 min, was measured by the loss of enzymically assayable pyruvate from the medium. The K_i values for α -cyano-4-hydroxycinnamate calculated by least-squares regression were $0.32 \pm 0.04 \mu\text{M}$ for control incubations ($\bullet$), $1.05 \pm 0.11 \mu\text{M}$ in the presence of 10 mM-L-lactate ($\blacktriangle$) and $2.39 \pm 0.21 \mu\text{M}$ in the presence of 1 mM-monochloroacetate ($\blacksquare$). The respective V_{max} values of transport were 44.8 ± 2.3 , 31.6 ± 1.2 and 27.3 ± 0.8 nmol/min per mg respectively.

presence of 4 mM-pyruvate the $K_i \pm \text{s.e.}$ for α -cyano-4-hydroxycinnamate in uncoupled mitochondria at pH 7.0 was $0.32 \pm 0.04 \mu\text{M}$ ($n = 8$) and the $V_{max} \pm \text{s.e.}$ for transport 44.8 ± 2.3 nmol/min per mg of protein. In the presence of 1 mM-monochloroacetate the values were $2.39 \pm 0.21 \mu\text{M}$ ($n = 7$) and 27.3 ± 0.8 nmol/min per mg, whereas in the presence of 10 mM-lactate the K_i was $1.05 \pm 0.11 \mu\text{M}$ ($n = 7$) and the V_{max} 31.6 ± 1.2 nmol/min per mg. Thus both L-lactate and monochloroacetate are competing with α -cyano-4-hydroxycinnamate for its binding site, as well as inhibiting the carrier by competing with pyruvate. The actual magnitude of the effects are of the order expected from the exchange data of Table 1. A similar effect is shown in Fig. 7, where the effect of monochloroacetate on the inhibition of pyruvate oxidation by compound UK 5099 [α -cyano- β -(1-phenylindol-3-yl)acrylate] in heart mitochondria is demonstrated. Like α -cyano-4-hydroxycinnamate (Halestrap & Denton, 1975), this compound was shown to have no inhibitory effect on pyruvate dehydrogenase at the concentrations required to inhibit pyruvate transport totally. At low inhibitor concentrations (100 nM) inhibition is relieved by 2 mM-monochloroacetate, but can be restored by addition of a higher inhibitor concentration (1 μ M). Lactate at 10 mM had very similar effects to 2 mM-monochloro-

Fig. 7. Effect of monochloroacetate on the inhibition by compound UK 5099 of pyruvate-dependent O_2 uptake by rat heart mitochondria

Pyruvate-dependent O_2 uptake was measured as described in the legend to Table 2. Additions were made as indicated. Rates of O_2 uptake are expressed as ng-atoms of O_2 /mg of mitochondrial protein. Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine.

acetate, as would be expected. Further evidence that α -cyano-4-hydroxycinnamate and its derivatives attack an essential thiol group on the inside of the membrane comes from the dependence of the K_i on the pH gradient across the mitochondrial membrane, which is reported in the following paper (Halestrap, 1978).

Role of the pyruvate carrier in the transport of ketone bodies

Both acetoacetate and DL- β -hydroxybutyrate are substrates for the pyruvate carrier, the former with quite high affinity (apparent K_m 0.56mM) and the latter with lower affinity (apparent K_m 5.6mM). However, this does not imply that entry into the mitochondria can only be achieved on the carrier. The experiments reported in Table 2 show that the oxidation of DL- β -hydroxybutyrate and acetoacetate by rat heart mitochondria is only partially inhibited by compound UK 5099 under conditions where pyruvate oxidation was almost totally abolished. Thus both these metabolites would appear to be able to cross the mitochondrial membrane without the pyruvate carrier. The uptake of DL- β -hydroxybutyrate was measured radioactively and was shown to be only partially inhibited by compound UK 5099 (20 μ M) (Fig. 8). However, incubation of mitochondria with 1.0mM-acetoacetate for 3 min followed by centrifugation through oil into 2% (w/v) HClO₄ containing 50% (w/v) glycerol and assay of the pellet for acetoacetate allowed measurement of the uptake of acetoacetate. Details of the method used are the same as described previously (Halestrap *et al.*,

Fig. 8. Effect of compound UK 5099 on the uptake of DL- β -hydroxybutyrate by rat liver mitochondria

Uptake was studied at 7°C and driven by an artificially induced pH gradient. The technique used was identical with that of Fig. 5, except that uptake was initiated by addition of DL- β -hydroxy[U-¹⁴C]butyrate (0.4 μ Ci/ μ mol) to a final concentration of 0.15mM, and terminated by rapidly centrifuging at 10000g for 1 min at 0°C in an Eppendorf 3200 centrifuge. Where added (●) compound UK 5099 was present at 20 μ M and preincubated with the mitochondria for 30s before addition of substrate. The rates of uptake calculated by first-order kinetic analysis were 0.56 nmol/min per mg (control) and, in the presence of compound UK 5099, 0.45 nmol/min per mg.

Table 2. Effect of compound UK 5099 on the oxidation of pyruvate and ketone bodies by rat heart mitochondria. Mitochondria (approx. 1mg of protein/ml) were incubated in medium (125mM-KCl/20mM-Tris/HCl, pH 7.2) at 30°C in a water-jacketed oxygen electrode chamber in the presence of 0.2 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. After a basal O₂-uptake rate was established, inhibitor was added to 2 μ M and the new rate measured. Values are representative of at least four experiments in all of which similar patterns of inhibition were evident.

Substrate	Concentration (mM)	Rate of O ₂ uptake (ng-atoms of O/min per mg)	
		Control	+Compound UK 5099
Pyruvate	0.25	160	20.1
	3.0	180	27.2
Acetoacetate	0.25	58.0	26.0
	3.0	93.5	67.2
DL- β -Hydroxybutyrate	0.25	6.4	2.4
	3.0	59.8	45.0

1974). Under these conditions the uptake of acetoacetate decreased from 1.86 ± 0.4 to -0.1 ± 0.41 nmol/mg in the presence of 0.5mM- α -cyano-4-hydroxycinnamate (values given as means $\pm$ S.E.M. of four observations). Problems were experienced in these experiments, since acetoacetate entering the mitochondria was rapidly reduced to β -hydroxybutyrate, which could then leave the mitochondria. Use was made of this to study the effects of a pyruvate carrier inhibitor on acetoacetate transport into the mitochondria. Mitochondria (approx. 4mg/ml) were incubated at 20°C for 4 min in 125mM-KCl/20mM-Mops, pH 7.0, containing 0.25mM-acetoacetate. Incubation was stopped by addition of HClO₄ to 2% and after centrifugation the β -hydroxybutyrate in the supernatant assayed. The output in 4 min dropped from 78.0 ± 2.7 to 45.9 ± 1.6 nmol/mg (means $\pm$ S.E.M. of four values) in the presence of 20 μ M-compound UK 5099. Measurement of H⁺ fluxes associated with pyruvate and ketone-body entry into mitochondria were performed as described by Halestrap (1975). Here, too, at low concentrations (0.50mM) of ketone bodies the inhibition of H⁺ flux by 10 μ M-compound UK 5099 was not more than 50% compared with about 90% for pyruvate and at higher concentrations (above 2mM) high rates of H⁺ flux were observed, which were not inhibited by compound UK 5099.

These results taken together suggest that at low concentrations of ketone bodies the pyruvate carrier may be an important, but not essential transport mechanism. Acetoacetate will use this with much greater ease than β -hydroxybutyrate. The proposal of Land & Clark (1974) that inhibition of mitochondrial ketone-body transport is important in the pathology of phenylketonuria and maple-syrup urine disease would seem unlikely in the light of these results. Indeed the data of Land & Clark (1974) suggest that pyruvate and β -hydroxybutyrate do not enter the mitochondria on the same carrier, since the inhibition of the oxidation of β -hydroxybutyrate by α -cyano-cinnamate requires a concentration of inhibitor a thousand times higher than that required to inhibit pyruvate oxidation in the same mitochondria. Far more likely to be of importance is the inhibition of pyruvate transport caused by phenylpyruvate and 2-oxo-4-methylpentanoic acid (Halestrap *et al.*, 1974; Clark & Land, 1974). Of greater interest is the fact that gluconeogenesis is normally high when ketogenesis is enhanced (see Exton, 1972). Under these conditions, exchange of pyruvate with ketone bodies may allow an enhanced rate of pyruvate transport into the mitochondria. This may be important if pyruvate transport is rate-limiting for gluconeogenesis, as is suggested in the following paper (Halestrap, 1978). To demonstrate that such an effect is feasible, the experiments of Fig. 9 and Table 3 were performed. Mitochondria were preloaded with β -hydroxybutyrate and then the rate of pyruvate transport was determined (Fig. 9). β -Hydroxybutyrate stimulated the rate of pyruvate transport 3-fold. Table 3 shows that if pyruvate transport is made rate-limiting by addition of $50\mu\text{M}$ - α -cyano-4-hydroxycinnamate under conditions of pyruvate metabolism

then addition of 0.5mM -octanoate (which greatly enhances ketone-body production) stimulates pyru-

Fig. 9. Effect of DL- β -hydroxybutyrate on the rate of pyruvate uptake by rat liver mitochondria. Uptake of pyruvate was studied at 7°C as described in Fig. 5, except that uptake was initiated by addition of pyruvate (0.25mM) to medium containing approx. 4mg of mitochondrial protein which had been preincubated at 7°C for 5min in the presence (▲) or absence (■) of 1mM -DL- β -hydroxybutyrate. Rates of uptake calculated by first-order kinetic analysis were $0.86\text{nmol/min per mg}$ in the absence of DL- β -hydroxybutyrate and $2.58\text{nmol/min per mg}$ in its presence.

Table 3. Effect of α -cyano-4-hydroxycinnamate and octanoate on pyruvate metabolism and ketone-body production by rat liver mitochondria

Mitochondria (approx. 3mg/ml) were incubated with shaking in 1ml of medium (125mM -KCl/ 20mM -Tris/HCl/ 10mM - KHCO_3 / 2.5mM -potassium phosphate/ 4.0mM -sodium pyruvate) at 37°C in sealed incubation flasks for 15min . $\text{H}^{14}\text{CO}_3^-$ was present at $1\mu\text{Ci/ml}$, and where indicated octanoate was added to 0.5mM and α -cyano-4-hydroxycinnamate to $50\mu\text{M}$. The incubation was terminated by addition of 0.1ml of 20% HClO_4 . After centrifugation the supernatant was analysed for pyruvate, acetoacetate, β -hydroxybutyrate and acid-stable ^{14}C as described under 'Methods'. Total pyruvate metabolism was calculated from the loss of pyruvate from the medium and pyruvate oxidation as the rate of this minus the rate of pyruvate carboxylation (Walter & Stucki, 1970; Stucki *et al.*, 1972). Values are given as the mean $\pm$ S.E.M. of four observations on the same batch of mitochondria. The statistical significance calculated by Student's *t* test are $\dagger P < 0.01$ compared with control without α -cyano-4-hydroxycinnamate, $* P < 0.01$ compared with control without octanoate.

Parameter values (nmol/min per mg)	Control	α -Cyano-4- cinnamate	Octanoate	Octanoate + α -cyano- 4-hydroxycinnamate
Pyruvate carboxylation	22.2 ± 0.1	$5.3 \pm 0.1\dagger$	$33.2 \pm 1.0^*$	$12.5 \pm 0.5^{*\dagger}$
Pyruvate uptake	40.8 ± 0.1	$11.2 \pm 0.6\dagger$	$38.3 \pm 0.2^*$	$14.5 \pm 0.4^{*\dagger}$
Pyruvate oxidized	18.6 ± 2.0	$5.9 \pm 0.7\dagger$	$5.2 \pm 1.1^*$	$2.0 \pm 0.2^{*\dagger}$
Acetoacetate output	4.5 ± 0.2	$1.3 \pm 0.6\dagger$	$13.8 \pm 0.3^*$	$11.3 \pm 0.7^*$
β -Hydroxybutyrate	1.7 ± 0.15	$0 \pm 0.20\dagger$	$3.9 \pm 0.5^*$	$6.8 \pm 0.5^{*\dagger}$
Total ketone-body output	6.2 ± 0.3	$1.3 \pm 0.5\dagger$	$17.7 \pm 0.5^*$	$18.0 \pm 1.2^*$

Fig. 10. Effect of pH on the rate of pyruvate transport and the K_t for α -cyano-4-hydroxycinnamate in rat liver mitochondria metabolizing pyruvate

Pyruvate metabolism was measured at 37°C under uncoupled conditions as described in the legend to Fig. 6. The pH and concentration of α -cyano-4-hydroxycinnamate were varied as indicated. The solid lines represent the Dixon plot best fitting the data. Where rates at zero inhibitor concentrations do not fall on the straight line they are connected by the broken lines.

vate metabolism. Since octanoate has no effect on the actual rate of pyruvate metabolism in the absence of inhibitor (although as expected it enhances the carboxylation and depresses the oxidation of pyruvate), the effect of octanoate in the presence of inhibitor is best explained by the stimulation of pyruvate transport brought about by ketone-body exchange.

Regulation of pyruvate transport by pH changes

We may conclude that one way of regulating pyruvate transport is through the rate of ketone-body production. Another method that can be predicted from the properties of the carrier would be through changes in pH. That such a process can occur under metabolizing conditions is shown in Fig. 10. In these experiments pyruvate metabolism in uncoupled heart mitochondria was inhibited by increasing additions of α -cyano-4-hydroxycinnamate. From the Dixon plots presented it would appear that

pyruvate transport only limits metabolism at pH values below 7. Above that pH, the zero inhibitor rates of metabolism do not fall on the straight line, indicating that under these conditions pyruvate transport is not rate-limiting. However, extrapolation of the Dixon plot to zero inhibitor concentration allows an estimate of V_{max} for transport at the various pH values used. These experiments show that in mitochondria oxidizing pyruvate in the uncoupled state, the V_{max} for transport is highly pH-dependent, whereas the K_t for α -cyano-4-hydroxycinnamate does not change with matrix pH (as distinct from the pH gradient across the membrane). The lack of effect of pH of the K_t for α -cyano-4-hydroxycinnamate is to be expected, since its reaction with an -SH group does not involve uptake or release of H^+ . Thus only the rate constant and not the equilibrium constant is pH-dependent (A. P. Halestrap, unpublished work; Holmes, 1975). The increase in V_{max} with increasing pH is very similar to that for pyruvate efflux from preloaded mitochondria (Fig. 4), giving an approximate pK of 8.1 and a V_{max} of approx. 600 nmol/min per mg of protein (the method does not allow an accurate estimate). This V_{max} is of the order expected from the V_{max} of 3.31 at 0°C and the activation energy of 113 kJ/mol.

General conclusions

The data in the present paper indicate that the kinetics of pyruvate transport across the mitochondrial membrane is highly dependent on the pH and the availability of an exchangeable ion (Figs. 1–4). The maximum rate of transport in the absence of such an exchangeable ion is substantially less than when an exchangeable ion is present. The nature of the exchangeable ion only affects the K_m but not the V_{max} for transport. These data allow the conclusion that pyruvate transport occurs with H^+ rather than in exchange for OH^- . Observations on the nature of the inhibition by α -cyano-4-hydroxycinnamate and its analogues suggest that inhibition occurs in two phases, binding to the carrier at the substrate site and then a reversible reaction with an -SH group. Preincubation of mitochondria with inhibitor leads to non-competitive inhibition, because the inhibitor traps the carrier on the inside of the mitochondria where it is not exposed to pyruvate. However, when a competing substrate can enter the mitochondria, displacement of inhibitor can occur (Figs. 6 and 7). The similarity of α -cyano-4-hydroxycinnamate to the enol form of pyruvate has been pointed out on previous occasions (Halestrap & Denton, 1974; Halestrap, 1975) and it may be this feature that allows the inhibitor to bind to the pyruvate site before reaction with the -SH group occurs. The competitive inhibition observed by Paradies & Papa (1975) may be explained by the technique they used to

Scheme 2. Proposed mechanism for the pyruvate carrier

study transport, since mitochondria only experienced a brief (15s) exposure to pyruvate and inhibitor simultaneously, during which time only the non-covalent binding of inhibitor would be observed (Fig. 5).

The specificity of the pyruvate carrier has been well studied (Halestrap, 1975; Paradies & Papa, 1975, 1976) and the following general requirements for transport can be identified. The best substrates are α -substituted short-chain aliphatic acids where the substitution is either an oxo group (as in pyruvate) or an electrophilic atom such as chloride, fluoride or cyanide (as in monochloroacetate). If the substitution is OH^- (as in lactate), the affinity is considerably decreased. Substitution in the β position (as in acetoacetate) is almost as effective and follows the same rules. These observations, coupled with the proposed method of inhibition of α -cyano-4-

hydroxycinnamate, suggest a mechanism of transport summarized in Scheme 2. This scheme suggests that oxo acids are transported as thiol-addition compounds, of which substrates such as monochloroacetate are analogues. The O^- formed in the addition is stabilized by a group ' AH^+ ' on the carrier which may also interact with the electrophilic chloride of monochloroacetate (c). The mode of inhibition by α -cyano-4-hydroxycinnamate fits very well with this scheme (b).

The involvement of the pyruvate carrier in the physiological transport of ketone bodies is hard to assess. The carrier is not essential, since diffusion of the free acid across the membrane is possible, but as pointed out by Paradies & Papa (1975), under ketogenic conditions such as starvation and diabetes, the number of ketone bodies is sufficiently high to allow a substantial rate of transport on the carrier. This may

be of particular importance in the liver, where, under ketogenic conditions, gluconeogenesis may occur at a high rate and rapid pyruvate entry into the mitochondria is required. The possibility of exchange of pyruvate with ketone bodies will fulfil this requirement. In the following paper it will be shown that pyruvate transport may also be stimulated by an increase in the matrix pH brought about by the action of glucagon (Halestrap, 1978). It is suggested that pyruvate transport may limit the rate of gluconeogenesis and that glucagon stimulates gluconeogenesis by a stimulation of pyruvate transport. Thus an understanding of the effects of pH and ketone bodies on pyruvate transport may be of key importance in our understanding of the control of gluconeogenesis.

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